THE WHOLE-BODY PROTEIN TURNOVER RESPONSE TO THE INGESTION OF INTRINSICALLY LABELED EGGS AT REST AND AFTER ENDURANCE EXERCISE

BY

JUSTIN THOMAS PAREL

THESIS

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Adviser:

Assistant Professor Nicholas A. Burd

ABSTRACT

A large focus of post-exercise recovery has been on the ingestion of dietary or supplemental protein to maximize skeletal muscle protein synthesis to facilitate remodeling (e.g., growth and repair of muscle). However, considerably less attention has been paid to the repletion of exercise-induced oxidative losses at the whole-body level. Body protein pools are continuously in flux and contribute protein for oxidative purposes during rest and exercise, and therefore have a recovery cost beyond the muscle-specific pool. The purpose of this thesis is to examine whole protein metabolism to prolonged endurance exercise, followed by the ingestion of a mixed macronutrient beverage that included moderate amounts of dietary protein in the form of intrinsically labeled egg protein in trained males. Specifically, we sought to examine dietary protein digestion and absorption kinetics, leucine oxidation, and postprandial net leucine balance at rest and after a bout of moderate-intensity running exercise. Seven trained young males were studied after receiving a primed, continuous infusion of L-[1-^{13}C]leucine and ingesting a beverage containing 18 g of egg protein intrinsically labeled with L-[$^{2}H_3$]leucine after 1 hour of treadmill running at 70% VO_{2peak}. This thesis will serve to examine the subsequent whole-body response to a novel exercise and recovery protocol, as well as provide insight and practical implications related to these findings.

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The maintenance and remodeling of skeletal muscle are profoundly important throughout the lifespan. Skeletal muscle is a dynamic, metabolically active tissue that serves a multitude of functions, including locomotion, coordinated contraction, and the regulation of blood glucose and lipids. As well, the robust metabolic demands of skeletal muscle make it a predominant driver of basal metabolic rate [21], thus making the improvement of skeletal muscle an important factor in bettering quality of life and health outcomes.

Exercise and dietary protein consumption serve as two of the most influential stimuli capable of modulating skeletal muscle. The physiological stresses of exercise serve to disturb homeostasis and drive a powerful metabolic response within the working muscle. Major energy producing metabolic processes, such as the tricarboxylic acid (TCA) cycle, beta oxidation, and the redox reactions of the electron transport chain occur within the mitochondria of muscle cells. These processes act to break down both endogenous and exogenous substrates and replete energy required for intense physical activity in order to meet the burden of exercise. The ingestion of dietary protein serves to offset both basal losses of nitrogen and losses accumulated during physical activity. Protein recommendations for athletes generally suggest an increased need for protein consumption in order to improve postprandial net protein balance, and thus incite the synthesis and repair of old and damaged muscle [36, 40, 41].

Exercise presents a number of aversive stimuli that stress skeletal muscle tissue and influence the subsequent metabolic consequences, including depletion of muscle and liver glycogen [12], loss of body fluids and electrolytes [26], and modulated digestion and absorption

of dietary protein [55]. The cumulative stresses caused by exercise stimulate high rates of protein turnover [16], which is characterized by the continuous repair, remodeling, and renewal of damaged body proteins by the flux of free amino acids through the coordination of protein synthesis and protein breakdown. During prolonged exercise, body proteins are partitioned toward energy producing processes as a means to prioritize fuel stores for sustained contraction [46, 63]. As a result, prolonged exercise has been associated with increased leucine oxidation [27, 33, 43] and whole-body protein turnover. In comparison to fat and carbohydrate oxidation, amino acids do not contribute nearly as much energy for performance purposes [2]. However, despite their smaller contribution, amino acid oxidation has been shown to markedly increase during moderate intensity exercise [23, 46]. An important distinction is that dietary protein supplies precursors for the synthesis of all body proteins and is not relegated solely to the recycling of contractile (myofibrillar) or energy-producing mitochondrial proteins, which are commonly examined in exercise studies. As such, measurement of turnover at the whole-body level is not tissue-specific. The importance and purpose of habitual turnover is to ensure that tissue quality is maintained.

The catabolism of amino acids during exercise create a demand for proper postexercise recovery strategies in order to repair and remodel damaged body proteins. Historically, endurance athletes have placed emphasis on glycogen restoration and appropriate hydration strategies to improve performance [11]. However, there has been substantially less concern for the ingestion of dietary protein as a postexercise recovery strategy, despite the considerable body of literature supporting an increase in amino acid sensitivity, and the benefit of dietary protein for offsetting oxidative losses of amino acids during exercise [10, 44, 47]. Because the adaptive response of skeletal muscle to exercise results in its repair and remodeling, effective recovery strategies are

more likely to allow for the expression of improved fitness and an enhanced capacity to handle future physiological stress.

It has been firmly established that the ingestion of dietary protein in the recovery period following a bout of exercise is important for the remodeling of skeletal muscle [9, 35, 48]. While modest amounts of protein (~20-25 g) are capable of near-maximally stimulating muscle protein synthesis after resistance exercise [35, 62], it is not yet well-understood how a similar amount of protein influences the muscle or whole-body synthesis response. Additionally, to what degree endurance exercise influences availability of dietary amino acids during the recovery period is unknown. The purpose of this literature review is to serve as a framework to better understand the whole-body protein response at rest and after endurance exercise. It will provide an overview of whole-body protein metabolism and the physiological response during the postabsorptive and postprandial phases of rest and recovery from endurance exercise. Subsequently, this thesis will provide insight into our research study examining the whole-body metabolic response to the ingestion of intrinsically labeled egg proteins at rest and after endurance exercise, and conclude with a detailed discussion pertaining to the results and practical outcomes of this research.

1.2 Overview of Whole-Body Protein Turnover

Body proteins are constantly in flux, dynamically undulating between synthesis and breakdown [42]. The postabsorptive (fasted) state is typically characterized by rates of muscle protein breakdown exceeding those of muscle protein synthesis, with roles reversing following consumption of dietary protein. The importance of this undulation is to ensure that old and damaged proteins are continuously renewed, thereby maintaining the structural and physiological integrity of these proteins. Predictably, exercise physiology research has a vested interest in the

examination of muscle protein turnover, as this provides tissue-specific insight to muscular adaptations, such as contractile protein remodeling and mitochondrial biogenesis. However, whole-body protein turnover is unique in that it is, by definition, a non-specific measurement of amino acid metabolism. Whole-body turnover studies do not allow us to quantify protein metabolism of specific organs or tissues [50]. It is also important to note that rates of turnover are tissue-dependent [50, 61]. This suggests that whole-body turnover is the aggregated response of several amino acid pools. Because the metabolic demand of tissue turnover extends beyond skeletal muscle, the consideration of other pools should not be overlooked.

In the context of athletics, the importance of developing a more critical understanding of the whole-body response to exercise is to ensure appropriate recovery strategies. Our current understanding of muscle protein turnover suggests that modest amounts (~20-25 g) of protein following exercise are capable of near-maximally stimulating the muscle protein synthetic response [35, 62]. However, the limitation to this assumption is that it either negates consideration of the need to replenish other body protein pools during exercise, or it suggests that muscle is the final pool to be replenished by protein ingestion, and therefore no further consumption is necessary. Like muscle protein turnover, the goal of proper postexercise recovery to offset whole-body protein losses should be to achieve a positive net balance.

It has been demonstrated that a small protein dose is capable of improving whole-body protein balance; A 2002 study by Levenhagen et al. showed that participants achieved a slightly improved net balance and an increase in whole-body protein synthesis following 60 minutes of recumbent biking at 60% of VO_{2max} with the ingestion of only 10 g of protein, while Lunn et al. saw a negative net balance after the consumption of 16 g of milk protein following 45 minutes of treadmill running at 65% of VO_{2max} [29, 30]. Both of the previous studies show improvements in

net balance after exercise and protein ingestion. However, it is important to recognize that in the presence of a suboptimal protein dose, it is not possible to maximally restore oxidative losses of amino acids and achieve a positive net balance. This was further evidenced by Koopman et al., who showed a net negative protein balance with a carbohydrate only beverage after exercise versus protein (33 g) and protein plus leucine [24]. At this time, we do not have definitive information on how much protein is required to maximally stimulate whole-body protein synthesis. Data by Kim et al. has shown a linear response to whole-body net balance when comparing 40 and 70 g of protein in rest and exercise groups [22], despite 70 g of protein being nearly two-to-three-fold greater than what has been shown to maximally stimulate muscle protein synthesis [35]. This data reasonably suggests that the increase in whole-body protein synthesis may be reflecting protein synthesis in other tissues, such as the gut [15], despite a maximally stimulated muscle protein synthetic response. As well, data by Nair et al. have quantified the contribution of muscle protein synthesis as 25-30% of whole-body protein synthesis, arguing that liver and splanchnic turnover may account for a far greater proportion due to their faster turnover rates [37]. With this in mind, an argument can be made that individuals performing endurance exercise should be accounting for the repletion of whole-body oxidative losses in order to best recover from exercise.

1.3 Measurement of Whole-Body Protein Turnover

There are a number of ways in which whole-body protein metabolism has been measured, including precursor methods, end-product methods, and kinetic analysis [61]. The measurement of whole-body protein turnover has traditionally been performed most effectively with the assistance of stable-isotope amino acid tracers. Earlier research utilized radioactive isotopes such as L-[U-^{14}C]lysine to measure the rate of whole-body turnover [60]; however, these have been

largely phased out in exchange for the use of stable-isotopes, which have proven to be much safer for human research. Popular tracers used in whole-body metabolic research include [^{15}N]glycine, L-[1-^{13}C]leucine, and L-[$^{2}H_5$]phenylalanine [58]. The use of L-[1-^{13}C]leucine is considered the reference method to obtain fair estimates of whole-body protein metabolism in most physiological conditions [58].

The use of stable-isotope methodology allows for precise measurement of whole-body metabolism by examining the flux of a given essential amino acid. This is possible because stable-isotope tracers are a "heavier" version of amino acids that already exist prevalently in the body; for example, the most commonly occurring carbon in the body is ^{12}C, at nearly 99% natural abundance. ^{13}C, a stable isotope of carbon, exists with a natural abundance of ~1%. As well, ^{13}C contains seven neutrons instead of its counterpart's six neutrons, and therefore has an extra atomic unit of mass. The use of a primed, continuous infusion of one of these labeled amino acids causes its systemic increase, or 'enrichment," and attempts to increase the naturally occurring label by anywhere from 1-5%. Once this artificial enrichment occurs and we can examine the flux of the amino acid, we are able to effectively measure the tracer in comparison to the tracee (the naturally occurring, unlabeled amino acid). The benefit of stable isotopes in metabolic research is that the label incorporation can be identified in various pools, including body fluids, body proteins, and even CO_2 from expired breath, making it possible to more conclusively determine the metabolic fate of the tracer. However, this model only works based on the assumption that tracers are treated identically to their naturally occurring counterparts.

Whole-body turnover studies most commonly utilize the measurement of leucine kinetics, and thereby L-[1-^{13}C]leucine stable isotopes. Leucine and the other branched-chain amino acids are metabolized primarily in extrahepatic tissues, and are therefore found in high abundance within

skeletal muscle [1, 39, 59]. It is within the intracellular space that leucine transaminates into alpha-KIC, a product that is released into circulation and can be used to indirectly measure intracellular L-[1-^{13}C]leucine enrichments without the need for muscle tissue collection [32]. Using a leucine tracer also allows for the calculation of oxidation and synthesis rates, and thereby calculation of turnover at the whole-body level [32]. However, the traditional use of stable isotopes for whole-body turnover measurements can become dramatically more challenging when attempting to accurately assess the influence of ingested dietary derived protein and the subsequent anabolic response.

The challenge of measuring the digestion and absorption kinetics of free or protein-bound amino acids manifests itself in the differential digestive responses of free amino acid tracers and those derived from ingested foods [7]. Due to these differences in the digestive response, digestion and absorption kinetics cannot be appropriately assessed for labeled free amino acids ingested with a bolus of dietary protein [7, 8]. To ameliorate this issue, researchers have turned to the development of intrinsically labeled food proteins as a way to reliably assess postprandial whole-body turnover. Intrinsically labeled proteins are developed by incorporating labeled amino acids directly into the matrix of dietary protein sources. Most notably, this has been performed via intravenous infusion of stable isotopes into dairy cows and through the feeding of labeled amino acids to chickens [5, 17, 52, 53]. The subsequent outcome has introduced the incorporation of protein-bound labeled amino acids directly in the food matrix of milk and eggs, which consequently may be consumed for use in metabolic research.

A major limitation of traditional stable isotope models is that researchers often orally administer free amino acids with a continuous infusion of an amino acid tracer, a method that conflicts with the fact that most individuals consume dietary amino acids in their intact form.

Therefore, observed leucine kinetics may not be as representative of a normal response to food ingestion [52]. Intrinsically labeled food proteins permit researchers to observe the interaction of amino acids in a more "standard" postprandial environment. As well, they can more reliably assess the speed of digestion and absorption of protein-bound amino acids based upon the rate of appearance of tracer in the plasma [7, 13]. This novel tool also allows for the determination of leucine oxidation, non-oxidative leucine disposal (NOLD), and even the proportion of amino acids that have been sequestered in splanchnic tissue after first-pass uptake [31].

1.4 Whole-Body Protein Turnover During Exercise

Prolonged endurance exercise generates a robust demand for fuel mobilization in order to sustain contraction. Of the three major energy producing substrates, aerobic exercise predominantly relies on the catabolism of glucose and lipid fuel sources during periods of intense energy demand. However, although not a primary fuel source, endurance exercise is more heavily reliant on the utilization of amino acids as an alternative fuel source [46] as compared to resistance exercise [49]. During endurance exercise, leucine oxidation increases dramatically [23, 46], diverting a fraction of available amino acids to be converted, via gluconeogenesis, into glucose for energy. The intensity of endurance exercise has been shown to influence the degree of leucine oxidation [23, 28, 34, 63, 64]. In a study performed by Lamont et al., the transition from rest to a moderate intensity bout of steady-state exercise caused a near-doubling of leucine oxidation [28], which was consistent with data from the aforementioned exercise studies. Endurance exercise further exacerbates leucine catabolism when performed in a fasted state, even at low intensities, as represented by data from Knapik et al. [23]. Participants in this study performed low-intensity endurance exercise (45% VO_{2max}) in both the postabsorptive and fasted (3.5 days) states while

undergoing a primed, continuous infusion of L-[1-^{13}C]leucine. The researchers found that the duration of the fasted state substantially increased the rate of leucine oxidation as compared to a shorter postabsorptive exercise bout. Conversely, fed exercise has been shown to mitigate oxidative losses of protein when measured by both phenylalanine and leucine tracers [25]. In a novel study by Koopman et al., the ingestion of a protein and carbohydrate beverage during prolonged exercise decreased protein degradation by a magnitude of nearly 60%, and increased whole-body protein synthesis. For the athlete looking to maximize recovery from prolonged exercise should consider strategies that offset the catabolic effects of prolonged, moderate-intensity exercise.

1.5 Postabsorptive and Postprandial Protein Turnover

The postabsorptive state is generally initiated by an overnight fast (~8-10+ hours) and is most commonly characterized by an increased rate of protein breakdown and a decrease in protein synthesis [19, 23, 37, 38]. This is the result of a sustained period without exogenous protein to offset oxidative losses and promote a net positive anabolic environment. Leucine flux during the fasted state is an indicator of proteolysis in humans [37]. The consequence of a prolonged fasted state results in the mobilization of amino acids from endogenous protein pools for the purposes of oxidization or resynthesis. Data from Knapik et al. shows a comparison between postabsorptive and fasted state leucine flux and oxidation, suggesting that the greater the duration of time before protein ingestion, the higher the amount of potentially irreversible leucine turnover [23]. This is also shown to hold true with regard to the release of other amino acids from skeletal muscle during prolonged fasting [56]. The magnitude of amino acid oxidation during a prolonged fast is likely

exaggerated when compared to a more standard postabsorptive state, which one might experience in the time between dinner and breakfast, for example.

Exercise seems to further exacerbate the degree of whole-body protein losses when performed fasted. In the previously discussed study conducted by Levenhagen and colleagues, after 1 hour of continuous cycling, participants who received no postexercise recovery beverage showed a net negative protein balance when compared to those who consumed protein and carbohydrates [29]. Interestingly, Howarth et al. separately demonstrated similar whole-body losses after exercise, even in the presence of caloric repletion via carbohydrate beverage. When given carbohydrate-only beverages during the postexercise period, participants exhibited net protein loss, supporting the necessity for protein replenishment to properly offset exercise-related protein losses [20].

Postprandial protein turnover exhibits a dramatic shift in net balance at rest and after exercise, and a converse response to that displayed during postabsorptive fasting. At the whole-body level, the fasted state is primarily associated with increased protein breakdown and decreased synthesis, while fed state turnover shows a dichotomous increase in protein synthesis [18, 45] and decreased or unchanged breakdown [8, 22], which may be further improved by exercise. The ingestion of protein during both rest and the postexercise recovery period serves a dual-purpose. First, free and dietary protein-derived amino acids elicit a robust increase in aminoacidemia. The availability of amino acids from a modest bolus of protein (in isolation or in a mixed macronutrient form) has been shown to be a major regulating factor of protein synthesis and oxidation [19, 57]. Secondly, the insulinotropic effect caused by the ingestion of amino acids [51] inhibits proteolysis [6] and further stimulates a protein synthetic response [4]. In the context of a mixed macronutrient setting, the co-ingestion of carbohydrates and protein further stimulates a hyperinsulinemia

response and may more effectively improve leucine balance after feeding through the inhibition of catabolic processes [14].

The effect of exercise on postprandial protein handling should not be dismissed. On numerous occasions, exercise has been shown to more dramatically improve the postprandial leucine response than in a rested condition [3, 25, 29], creating a positive net protein balance in the presence of protein after exercise. The achievement of positive net balance in the postexercise period may be resultant of a more dramatic blunting of protein breakdown rather than improvement of protein synthesis following exercise [19]. Therefore, the provision of an exogenous protein source in the postexercise period generates a large number of available circulating amino acids, and thus greater potential for the escalation of whole-body protein synthesis and an achieved positive whole-body net balance.

It is interesting to note that splanchnic hypoperfusion – the redistribution of blood from splanchnic tissue towards active muscle – has been shown to cause gut dysfunction in healthy men following a bout of endurance exercise [54]. The appearance of plasma intenstinal fatty acid binding protein (I-FABP), which is released from enterocytes during hypoperfusion, is used as a marker of gut damage. Furthermore, gut damage has been demonstrated to blunt the digestion and absorption of amino acids following exercise [55]. Van Wijck and colleagues investigated the impact of splanchnic hypoperfusion on digestion and absorption kinetics at rest and after a bout of resistance exercise, revealing a reduced postprandial rise in plasma 1-^{13}C phenylalanine after exercise [55]. This suggests a blunted rated of uptake in the postexercise period compared to rest. In the discussion of the endurance athlete, this is crucial to consider, as replenishment of oxidative losses during the postprandial period is a necessity to maximize protein repletion and recovery for subsequent performance. Athletes attempting to maximinze recovery may need to consider the

potential for exercise to blunt digestion and absorption when considering protein repletion strategies.

1.6 Conclusion

In conclusion, endurance exercise is an energy-intensive task that demands appropriate measures to best offset losses of body proteins accumulated during exercise performance. It is well-established that the effect of exercise stimulates an intramuscular catabolic response in order to provide energy for sustained work. Particularly, endurance exercise has a more profound capacity to mobilize amino acids as an alternative fuel than resistance exercise, and therefore necessitates the subsequent ingestion of dietary protein-derived or free amino acid sources to best replete amino acid losses. Dietary protein-derived and free amino acids are capable of not only stimulating an anabolic whole-body response, but may offset or improve protein losses generated during exercise. Classically, the study of whole-body protein turnover has been performed with the assistance of stable isotope amino acid tracers. However, the limitations of such methodology often relegate the study of whole-body turnover to the use of free amino acids as a fuel source during study interventions. Because intact protein sources most commonly constitute protein ingested in a standard diet, it is imperative to utilize novel research tools such as intrinsically labeled food in order to best examine the influence of bolus meal-like protein sources on digestion and absorption kinetics. To date, no study has utilized intrinsically labeled dietary proteins in order to investigate digestion and absorption kinetics and amino acid oxidation rates when comparing rest and exercise trials. Exploring these responses may further expand our ability to provide sound recommendations for endurance athletes seeking to improve both recovery from exercise and

subsequent performance. The following research study provided will seek to investigate these questions.

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CHAPTER 2: THE INGESTION OF A MODERATE AMOUNT OF PROTEIN ATTENUATES BUT DOES NOT RECOVER LEUCINE OXIDATIVE LOSSES AFTER ENDURANCE EXERCISE IN YOUNG MALES

2.1 Research Project Manuscript

Introduction

The physiological demands of endurance exercise create whole-body metabolic perturbations. These disturbances generate a need for fuel mobilization in order to provide the working muscle with energetic substrates. Prolonged, continuous endurance exercise lasting ≥ 1h induces a number of metabolic demands on the body, including depletion of carbohydrate fuel stores (e.g. muscle and liver glycogen) [9], loss of body fluids and electrolytes through sweat [19], altered dietary protein digestion and absorption kinetics [41], as well as damage and breakdown of body proteins [15]. Most athletes engaging in endurance-type training recognize the importance of hydration and postexercise carbohydrate intake to restore muscle and liver glycogen [6]. However, considerably less attention has been paid to the role of dietary protein to enhance post-endurance exercise recovery.

Whole-body protein turnover (WBPT) is characterized by the flux of free amino acids through coordination of protein synthesis (WBPS), breakdown (WBPB), and amino acid oxidation (AA_{ox}). Therefore, net balance is determined as the algebraic difference between protein synthesis and breakdown. This process ensures continuous repair, remodeling, and renewal of damaged body proteins. The performance of acute endurance exercise is marked by an increased use of amino acids (i.e. leucine) as an alternative fuel source, blunting their ability to repair body proteins during recovery, as well as reducing rates of protein synthesis as a means to prioritize fuel stores toward sustained contraction [31, 48]. Furthermore, endurance exercise appears to increase WBPB and

AA_{ox} [31]. Yet, the acute effects of endurance exercise on WBPS are less clear with previous reports showing decreased protein synthesis [31, 49] or no change [8, 29].

In the absence of dietary protein, postexercise protein balance has been shown to improve, favoring WBPS [12, 31] over WBPB [39]. Yet, existing data has revealed that, despite this upward trend following exercise, the consumption of a suboptimal amount of protein may insubstantially improve protein balance, thereby causing net balance to remain negative after exercise [20, 25, 29]. Therefore, postexercise ingestion of an optimal amount of protein is a critical strategy for offsetting exercise-induced protein loss and creating a positive net balance. Protein ingestion after exercise has been shown to improve WBNB [22, 24] potentially through the offsetting of oxidative losses to achieve positive nitrogen balance [29, 37]. This is crucial for the endurance athlete, as maintenance of the body protein pool following training sessions may have implications for future endurance exercise performances [33, 38].

Much of the focus for postexercise recovery has been on skeletal muscle protein metabolism, where relatively modest amounts of protein (~20 g) are capable of near-maximally stimulating muscle protein synthesis at rest [47], and after endurance exercise [32]. However, little evidence exists to determine the efficacy of this protein dose for improving whole-body net protein balance. Moreover, it is not known how endurance training influences the availability of dietary amino acids during the recovery period. To date, no study has compared the results of an exercise trial to a rested trial to determine the impact of an acute bout of endurance exercise on dietary protein digestion and absorption kinetics, leucine oxidation rates, and postprandial leucine balance. Accordingly, the objective of the present study was to examine how protein ingestion as part of a mixed macronutrient meal may replace exercise-induced leucine oxidative losses and enhance postprandial leucine balance following a bout of treadmill running in trained young men. We

hypothesized that – following an overnight fast – ingestion of a moderate dose of protein (18 g) after acute endurance exercise will improve whole-body protein balance at rest and during postexercise recovery. These findings will assist with the development of nutritional recommendations to provide endurance athletes with guidelines for optimal postexercise recovery.

Methods

Participants and ethical approval

Seven trained young men (25.6 ± 6.2 y; 72.4 ± 7.6 kg; VO_{2peak} = 61.7 ± 7.8 $ml \cdot kg^{-1} \cdot min^{-1}$; means ± SD) were included in the present study. Participants were active runners who engaged in endurance exercise 3-6 times per week and were recruited from various athletic clubs at the University of Illinois at Urbana-Champaign. All participants were considered healthy based on responses to a physical activity readiness questionnaire and a medical history form. Participants were informed of the study purpose, experimental protocol, and potential risks prior to providing written consent to participate. The study was approved by the University of Illinois at Urbana-Champaign Institutional Review Board and the University of Toronto Research Ethics Board and was conducted in accordance with the Declaration of Helsinki (revised version, October 2013, Fortaleza BRA).

Experimental design

A randomized crossover design was used for the present study. Prior to the infusion protocol, participants reported to the laboratory for familiarization with the exercise equipment and for VO_{2peak} assessment, as determined by graded treadmill exercise testing and gas flow analysis. In addition, body composition was measured by dual-energy X-ray absorptiometry

(Discovery QDR Series Bone Densitometer, software version 12.7.9, Hologic, Bedford MA). On another visit, participants reported to the laboratory to perform a habituation trial. During this trial, participants performed 60 min of treadmill running in order to determine treadmill settings for the experimental trial. Treadmill settings were adjusted during the first 5 min to elicit an intensity of 70% VO_{2peak} while running at 1% grade and maintained for 60 min. Participants were instructed to refrain from any form of vigorous physical exercise and to consume their typical diet for two days prior to the infusion trial.

Infusion protocol

Participants engaged in a rest and exercise infusion trial in random order (Fig. 1). The trials differed only in the inclusion of a 60-min bout of treadmill running at 70% VO_{2peak} and were separated by ≥ 7 days. On the morning of the infusion trial, participants reported to the laboratory at ~0700h following an overnight fast. A Teflon catheter was inserted in an antecubital vein for stable isotope infusion. Participants received priming doses of $NaH^{13}CO_2$ (2.4 μmol·kg^{-1}) and L-[1-^{13}C]leucine (7.6 μmol·kg^{-1}) prior to initiating a continuous infusion of L-[1-^{13}C]leucine (0.13 μmol·kg^{-1}·min^{-1}). A second Teflon catheter was inserted in a dorsal hand vein of the contralateral arm and placed in a heated blanket (60ºC) for arterialized blood sampling. Blood sampling occurred before (*t* = -180, -120, -90, -60, -30, and 0 min) and after (*t* = 30, 60, 90, 120, 150, 180, 210, 240 and 300 min) beverage ingestion to determine plasma insulin, intestinal fatty acid binding protein (I-FABP; surrogate marker of gut damage), and leucine concentrations, as well as plasma enrichments of the intravenous and oral leucine tracers. At similar time points as blood was drawn, breath samples were collected to determine [$^{13}CO_2$] enrichment. Additional blood and breath

samples were collected during exercise (*t*= -40, -20, and -5 min). VCO_2 was measured at *t* = -85, -40, 35, 95, 165, 275 min to determine rates of leucine oxidation.

Beverage preparation

At *t* = 0 min, participants ingested a beverage containing 18 g of egg protein intrinsically labeled with $_L$-[5,5,5-$^2H_3$]leucine, 60 g of carbohydrate (blend of 50% sucrose, 50% maltodextrin), and 17 g of fat in 400 ml of water. The $_L$-[5,5,5-$^2H_3$]leucine enrichment of the eggs was 21.2 MPE. Intrinsically labeled eggs were produced by the Poultry Research Farm, Animal Science Laboratory, University of Illinois at Urbana-Champaign, according to methods previously described [40].

Plasma extraction

Blood samples were collected into pre-chilled tubes containing EDTA and centrifuged (Allegra X-14, Beckman Coulter, Brea CA) for 10 min at 3600 rpm and 4ºC. Subsequently, the plasma was aliquoted and immediately stored at -80ºC.

Blood glucose, plasma insulin, and I-FABP concentrations

Blood glucose concentration was determined using a glucose/lactate analyzer (YSI 2300 Stat Plus, Marshall Scientific, Hampton NH). Briefly, following each blood draw, 25 µl of sample was drawn into the machine and the sample's analyte values were displayed and recorded. The blood glucose analyzer was automatically calibrated every 15 min while operating in run mode. Plasma insulin concentration was determined by solid two-site enzyme immunoassay (Mercodia Diagnostics, Uppsala SWE). To determine whether treadmill running increases markers of gut

damage, I-FABP concentration was analyzed by solid-phase enzyme immunoassay (Hycult Biotechnology, Uden, NLD).

Plasma leucine concentration

Plasma leucine concentration was determined by liquid chromatography tandem mass spectrometry (LC-MS-MS; 1290 HPLC, Agilent Technologies, Santa Clara CA; 5500 Q-Trap MS, Sciex, Framingham MA). Briefly, 20 µl of plasma was centrifuged for 10 min at 10,000 rpm and 4ºC, and the supernatant was dried under N_2 at 37ºC. The remaining residue was treated with 100 µl of 3.0 M HCl in *n*-butanol, dried under N_2, and reconstituted in 100 µl of acetonitrile:water+0.1% formic acid (20:80, v/v). Subsequently, 1 µl of sample was injected into an LC-MS-MS system and HPLC was performed on a 50 m column (Kinetex XB-C18, Phenomenex, Torrance CA) with 2.6 µm inner diameter and 3 mm film thickness. Flow rate was set to 450 $\mu l \cdot min^{-1}$ with a total run time of 4 min. Natural leucine and the internal standard ($_d$10-leucine, Cambridge Isotope, Tewksbury MA) were identified by a Q3 mass of 43 and 48.1 Da, respectively. Plasma leucine concentration was calculated based on the slope of a standard curve.

Plasma leucine enrichments

Plasma enrichments of the intravenous ($_L$-[1-^{13}C]) and oral ($_L$-[5,5,5-$^2H_3$]) leucine tracers were determined by GC-MS (5975C MSD, Agilent Technologies, Santa Clara CA) using previously published methods [2, 40]. Briefly, 200 µl of plasma was extracted using 1 ml of a single phase mixture of isopropanol:acetonitrile:water (3:2:2, v/v) and centrifuged for 10 min at 10,000 rpm and 4ºC. The supernatant was dried under N_2 and leucine was converted to its *tert*-butyldimethylsilyl (*t*-BDMS) derivative prior to GC-MS analysis. Subsequently, 3 µl of sample

was injected in a split mode (10:1) into the GC-MS system and electron impact gas chromatography was performed on a 60 m column (ZB-5MS, Phenomenex, Torrance CA) with 0.32 mm inner diameter and 0.25 mm film thickness, with an injection temperature and MSD transfer line of 230ºC and the ion source adjusted to 230ºC. The helium carrier gas was set at a constant flow rate (2 ml·min^{-1}). The temperature program was 5 min at 110^{0}C, followed by an oven temperature ramp of 5ºC·min^{-1} to 315ºC for the final 5 min. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in an *m/z* 50-800 scan range. Plasma leucine enrichments were determined by selective ion monitoring at *m/z* 302 and 303 for unlabeled and labeled L-[1-^{13}C]leucine, respectively; and *m/z* 302 and 305 for unlabeled and labeled L-[5,5,5-$^{2}H_3$]leucine, respectively.

Plasma α-[^{13}C]ketoisocaproate enrichment

Plasma enrichment of α-[^{13}C]ketoisocaproate (α-KIC) was measured by GC-MS (7890B GC, 5977A MSD, Agilent Technologies, Santa Clara CA) as a surrogate for intramuscular [45, 46] and hepatic [1] leucyl-transfer RNA labeling. Briefly, 100 µl of plasma was deproteinized and centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant was dried under N_2 and the remaining residue was extracted using *o*-phenylenediamine and dichloromethane. Following evaporation, α-KIC was converted to its *t*-BDMS derivative prior to GC-MS analysis. Subsequently, 1 µl of sample was injected in a pulsed-split mode (5:1) into the GC-MS system and gas chromatography was performed on a 30 m phenyl methyl silox column (HP-5MS, Agilent Technologies, Santa Clara CA) with 250 µm inner diameter and 0.25 µm film thickness, with an injection temperature and MSD transfer line of 250ºC. The helium carrier gas flow was set at a constant flow rate (1.2 ml·min^{-1}). The temperature program was 5 min at 120ºC, followed by an

oven temperature ramp of 10ºC·min^{-1} to 160ºC and 20ºC·min^{-1} to 290ºC. The post-run time was set to 300ºC for 2 min. The mass spectrometer was operated in positive electron impact mode in an *m/z* 50-300 scan range. Plasma α-KIC enrichment was determined by selective ion monitoring at *m/z* 232 and 233 for unlabeled and labeled α-KIC, respectively.

Breath [$^{13}CO_2$] enrichment

Breath enrichment of [$^{13}CO_2$] was analyzed using IRMS (ID-Microbreath, Compact Science Systems, Newcastle UK). Breath samples were analyzed sequentially with 3 technical replicates ordered in series. Atom percent (AP) of [^{13}C] was calculated from Pee Dee Belamite (PDB) values as AP[^{13}C] = 100/(1/((delta/1000+1)*R_{ref})+1, where R_{ref} = 0.0112372 for PDB.

Calculations

Whole-body leucine kinetics were estimated in non-steady state conditions using oral and intravenous administration of leucine tracers and were determined by modified Steele equations [34] as previously described [4, 10, 11]. Leucine oxidation was calculated using α-KIC as the precursor pool because α-KIC is the immediate precursor of leucine decarboxylation [46]. For the other fluxes, calculations were performed using plasma [^{13}C] and [$^{2}H_3$]leucine MPE as precursors

Total, exogenous, and endogenous leucine rate of appearance

Total leucine rate of appearance (Total Leu R_a) into circulation is the sum of the rate of entry of exogenous leucine (Exo Leu R_a), which is taken as an index of dietary protein digestion rate and the intravenously infused leucine (*ir*), and the rate of entry of endogenous leucine (Endo Leu R_a) derived from protein breakdown. These parameters, as well as the splanchnic extraction

of leucine (*Sp*; i.e., the fraction of dietary leucine taken up by the gut and the liver during its first pass) were calculated as follows:

$$Total\ Leu\ R_a = \frac{ir - pVC(t)dE_{iv}/dt}{E_{iv}t},$$
$$Exo\ Leu\ R_a = \frac{Total\ Leu\ R_a E_{PO}(t) + pVdE_{PO}/dt}{E_{Prot}},$$
$$Endo\ Leu\ R_a = Total\ Leu\ R_a - ir,$$
$$Sp = 100\left(\frac{Leu_{Pro} - AUC_{ExoLeuRa}}{Leu_{Pro}}\right),$$

where, pV (0.125) is the leucine pool size corrected for instant mixing. This constant is the same as used previously described [4, 10, 11]. *C(t)* represents the mean plasma leucine concentration between two sampling points. dE_{iv}/dt corresponds to time-dependent variations of plasma leucine MPE of the intravenous leucine tracer, and $E_{iv}t$ is the mean plasma leucine MPE derived from the intravenous tracer between two consecutive time points. $E_{po}(t)$ represents the mean plasma leucine MPE of the oral tracer between two time points, dE_{po}/dt is the time-dependent evolution of plasma leucine MPE of the oral tracer, and E_{Prot} corresponds to the oral tracer enrichment in dietary protein. Exo Leu R_a is calculated according to Proietto's modification of Steele's equations [30]. Leu_{Pro} is the amount of dietary leucine ingested. $AUC_{ExoLeuRa}$ represents the area under the curve for Exo Leu R_a (calculated by trapezoidal method). This corresponds to the amount of dietary leucine that appeared in the peripheral blood over the 300-min postprandial period.

Total leucine rate of disappearance, leucine oxidation, and non-oxidative leucine disposal

The total leucine rate of disappearance (Total leucine R_d*)* from the plasma corresponds to the sum of the fluxes of leucine oxidation (Leu Ox) and leucine utilized for protein synthesis (i.e., non-oxidative leucine disposal; NOLD). These parameters were calculated as follows:

$$Total\ Leu\ R_d = Total\ Leu\ R_a - pV\frac{dC}{dt},$$

$$Leu\ Ox = \frac{\dot{V_{CO_2}} E_{CO_2}}{k E_{13C-KIC}},$$
$$NOLD = Total\ Leu\ R_d - Leu\ Ox,$$

where, *dC/dt* corresponds to the time-dependent variation of plasma leucine concentration. E_{CO2} and $E_{13C\text{-}KIC}$ correspond to [$^{13}CO_2$] and α-KIC MPE, respectively. *k* is a correcting factor for the incomplete recovery of CO_2 in the breath (0.8), as previously described [4, 10, 11].

Net leucine balance

Postabsorptive leucine balance was calculated directly as the difference between the intravenously infused leucine and the amount of leucine oxidized over the 60-min postabsorptive period. Postprandial leucine balance was calculated as the difference between dietary leucine intake and the amount of leucine oxidized over the 300-min postprandial period. Total leucine balance was calculated as the sum of postabsorptive and postprandial leucine balance.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6.02, GraphPad software for Windows, San Diego CA). A within-subject repeated measures design was used for the present study. Differences in plasma concentrations and whole-body leucine kinetics were tested using a two-factor (treatment × time) analysis of variance (ANOVA) with repeated measures on time. The same analysis was performed to identify differences between postabsorptive and postprandial leucine balance. Where significant interactions were identified in the ANOVA, Tukey's post hoc test was performed to determine differences between means for all significant main effects and interactions. A Bonferroni-corrected t-test was used to identify differences in total leucine balance. All data are expressed as means ± SD. For all analyses, statistical significance was set at $P < 0.05$.

Results

Tracer enrichments

Figures and details concerning [^{13}C] and [$^{2}H_3$]leucine, α-KIC, and [^{13}CO2] enrichments are provided as Supplementary material with the online version of this paper.

Plasma metabolites

Blood glucose (Fig. 2A), plasma insulin (Fig. 2B), and plasma leucine (Fig. 3) concentrations increased (main time effect, $P < 0.01$) following protein ingestion, with no differences (glucose: treatment effect, $P = 0.72$; interaction, $P = 0.46$; insulin: treatment effect, $P = 0.78$; interaction, $P = 0.93$; leucine: treatment effect, $P = 0.15$; interaction, $P = 0.37$) between the rest and exercise conditions.

Exercise-induced gut damage and appearance of dietary amino acids

There was a main effect of time ($P < 0.01$), treatment ($P < 0.01$), and treatment × time interaction ($P < 0.01$) for plasma I-FABP concentration (surrogate marker of gut damage; Fig. 4) with values increasing during the exercise bout and returning to baseline ($P > 0.05$) within 60 min of recovery. I-FABP concentration was stable ($P > 0.05$) in the rest condition across time. Exogenous leucine rate of appearance (representing the appearance of dietary protein-derived leucine into the circulation; Fig. 5A) increased (main time effect, $P < 0.01$) after protein ingestion, with no differences (treatment effect, $P = 0.91$; interaction, $P = 0.92$) between conditions. The amount of dietary leucine that appeared in the circulation over the 300-min postprandial period was similar ($P = 0.82$) at rest and after exercise (62 ± 5 and 63 ± 4%, respectively).

Whole-body leucine kinetics

Endogenous leucine rate of appearance (representing the appearance of leucine derived from whole-body protein breakdown into the circulation; Fig. 5B) was suppressed ($P < 0.01$) after protein ingestion with no differences (treatment effect, $P = 0.50$; interaction, $P = 0.17$) between the rest and exercise conditions. There was a main effect of time ($P < 0.01$), treatment ($P = 0.01$), and treatment × time interaction ($P < 0.01$) for leucine oxidation (Fig. 6A) with greater rates during the exercise bout and returning to baseline ($P > 0.05$) immediately in recovery. Leucine oxidation was stable ($P > 0.05$) in the rest condition across time. There was a main effect of time ($P < 0.01$), treatment ($P < 0.01$), and treatment × time interaction ($P < 0.01$) for non-oxidative leucine disposal (i.e., whole-body protein synthesis; Fig. 6B) with lower rates during the exercise bout and for 180 min of recovery. Non-oxidative leucine disposal was unchanged ($P > 0.05$) in the rest condition across time.

Net leucine balance

There was a main effect of time ($P < 0.01$), treatment ($P < 0.01$), and treatment × time interaction ($P < 0.01$) for net leucine balance (Fig. 7). Postabsorptive leucine balance was more negative during the exercise bout compared to rest (-71.4 ± 9.1 vs. -24.2 ± 2.0 μmol·kg^{-1}, respectively). Postprandial leucine balance was enhanced in the rest and exercise trials (40.8 ± 10.2 and 47.4 ± 10.4 μmol·kg^{-1}, respectively) with no differences ($P > 0.05$) between conditions. Total leucine balance (calculated as the sum of postabsorptive and postprandial balance) was significantly lower ($P < 0.05$) during the exercise compared to the rest trial (-24.0 ± 13.7 vs. 16.6 ± 9.9 μmol·kg^{-1}, respectively).

Discussion

Dietary protein is an important factor in ensuring the maintenance of lean body mass. The protein content of a mixed meal functions to replenish postabsorptive and/or oxidative losses of this critical macronutrient [28]. Despite the preeminence of dietary protein for enhancing whole-body protein anabolism in trained young men [26], to the best of our knowledge, we present here the first data to address the impact of an acute bout of endurance exercise on postprandial leucine kinetics and net leucine balance. Specifically, we demonstrate that a moderate amount of protein within a mixed macronutrient beverage enhances postprandial leucine balance; however, the amount of protein consumed was not sufficient to fully replace exercise-induced oxidative leucine losses, which resulted in a negative total leucine balance during postexercise recovery. Moreover, despite significant increases in markers of gut damage during exercise, the appearance of dietary-derived leucine was not altered during recovery in our trained men.

In the present study, protein breakdown was unchanged, whole-body protein synthesis was suppressed, and leucine oxidation was increased during exercise. These data are in agreement with previous findings [5] and suggest that there is a decrease in protein synthesis during exercise that is related to the diversion of amino acids away from events supporting synthesis and toward events supporting muscle contraction (i.e., leucine oxidation [31, 48]). The increased use of leucine as an alternative fuel source during the exercise bout led to a significantly more negative leucine balance compared to rest.

The current study also sought to examine the effect of running exercise on a marker of gut damage: plasma I-FABP. It has been previously demonstrated that exercise-induced gut damage is caused by splanchnic hypoperfusion [41]. The increase in plasma I-FABP in the present study suggests our exercise model also induced gut damage in our trained athletes. Our data are in

agreement with previous findings [41], in which untrained men performed 60 min of cycling exercise at 70% of maximal workload capacity. Interestingly, when trained men performed similar exercise, a smaller magnitude of increase in I-FABP was observed [42], suggesting that training status may influence the gut damage response to exercise. Furthermore, the higher impact forces associated with weight bearing running in the present study may have contributed to a greater physical damage to gut tissue when compared to low impact, stationary cycling; this possibility remains to be determined, including whether I-FABP may be an appropriate surrogate marker of physical damage to the small intestines.

Exercise-induced gut damage has been associated with a blunted appearance of dietary-derived amino acids into the circulation [43]. In the present study, gut damage did not impair the appearance rate of dietary-derived leucine. The conflicting findings in exogenous amino acid appearance may be explained by several physiological mechanisms. In the previous study [43], the appearance rate of dietary-derived phenylalanine peaked rapidly (30 min) following protein ingestion and remained suppressed below resting rates for 60 min of recovery. In turn, peak rate of appearance coincided with the return of I-FABP to normal levels in the exercise condition. The correlation between I-FABP levels and *in vivo* exogenous amino acid kinetics implies that the extent of gut damage (as measured by I-FABP) is a good reflection of the impact of exercise on intestinal function with respect to dietary protein digestion. Alternatively, the attenuated exogenous rate of appearance may reflect greater splanchnic uptake of dietary amino acids. However, in the present study, peak exogenous leucine rate of appearance was delayed (120 min) following protein ingestion, with no differences observed between the rest and exercise conditions. Additionally, I-FABP levels returned to normal values within 60 min of recovery, which suggests that I-FABP was cleared from the plasma before the peak rate of appearance of dietary leucine.

Although we cannot determine if this was a resolve of the damage incurred during exercise and/or reflective of a greater splanchnic uptake, our results demonstrate that, despite increases in I-FABP during exercise and for 60 min of recovery, dietary-protein derived amino acid appearance was not impaired. The delayed rate of appearance was beneficial, in that it allowed time for the gut to repair prior to the release of amino acids into circulation.

An alternative explanation for the lack of augmented postprandial protein handling may be in part due to the tracer. In studies using phenylalanine as a tracer, first-pass splanchnic extraction of phenylalanine is ~45% [14]. In the present study, we utilized a protein intrinsically labeled with leucine, and first-pass splanchnic extraction of the leucine tracer was ~38%. Therefore, perhaps a similar response to dietary protein in the previous study [43] could have been observed if they used an amino acid tracer that was less extracted by the gut (i.e., leucine). Therefore, given the importance of exogenous amino acids to facilitate postexercise recovery [27], our data suggest that athletes need not be concerned with the impact of mild gut injury on the appearance of dietary protein-derived amino acids during recovery.

In the context of mixed meals, the hormone insulin is a major regulator of postprandial protein turnover primarily through its inhibitory effect on protein breakdown [3]. In the present study, whole-body protein breakdown was suppressed following mixed macronutrient beverage ingestion at rest and after exercise, suggesting that the postprandial protein breakdown response was not attenuated during postexercise recovery. These data are in agreement with previous findings [16], in which protein and carbohydrate co-ingestion reduced whole-body protein breakdown following 2 h of cycling. These data confirm the anti-catabolic effect of carbohydrate-induced hyperinsulinemia and are in agreement with previous findings [11].

The attenuated plasma leucine response observed in the present study may be attributed to the nutrient composition of the beverages. Non-protein energy sources (i.e., fat and carbohydrate) are known to decrease the rate of postprandial gastric emptying [7, 17]. Specifically, the presence of fat in the small intestine is the most potent inhibitor of gastric emptying [23]. This could explain the modest effect that protein ingestion had on plasma leucine concentration in the present study.

The postprandial availability of leucine, which is reflected by increases in plasma leucinemia, is the major regulating factor of postprandial whole-body protein synthesis and oxidation [13, 44]. Thus, in the absence of a robust increase in postprandial leucine concentration, it is apparent why protein ingestion failed to stimulate protein synthesis or oxidation in the present study.

Additionally, protein synthesis remained lower during postexercise recovery when compared to rest in the present study. These data agree with previous findings [16, 22], in which protein synthesis remained suppressed following endurance exercise, despite postexercise protein ingestion. The previous studies [16, 22] lacked a measurement of resting whole-body protein synthesis and could not resolve whether the suppression during recovery represented a return to baseline or a potential decrease below resting values. Hence, the literature seems to suggest that protein synthesis is either unchanged or potentially suppressed during recovery despite protein ingestion. In the present study, we were able to compare rates of postexercise rates of synthesis to pre- and intra-exercise values. This allows us to conclude that there is a carry-over effect of suppressed protein synthesis during exercise and into recovery, which ultimately attenuates the postprandial synthetic response to protein ingestion after exercise. However, despite no difference or a decrease in protein synthesis, dietary protein can still support greater rates of muscle protein

synthesis after endurance exercise that may not be captured by whole-body tracer methodologies [16, 22].

Appropriate protein ingestion is necessary to counterbalance postabsorptive amino acid losses. Furthermore, the exercise-induced changes in protein metabolism (i.e., oxidative leucine losses) highlight the importance of consuming dietary protein during recovery to provide the amino acid building blocks for the restoration of, and potential increase in whole-body protein (i.e. leucine) balance. In the present study, protein ingestion did not stimulate postprandial leucine oxidation, likely due to a muted plasma leucine response, which may explain why our participants were in a net positive leucine balance at rest. Conversely, total leucine balance was negative during postexercise recovery, likely due to an exercise-induced increase in leucine oxidation. These data suggest that a greater dose of protein or more frequent feedings may be required to ensure amino acid replacement following exercise in order to minimize the deleterious effects of oxidation and to maximize whole-body protein accretion. In the present study, we were able to account for oxidative leucine losses incurred during exercise to determine true net balance, which resulted in a negative total net balance during postexercise recovery.

It has recently been demonstrated that protein requirements are elevated in endurance athletes after exercise [18], likely due to an increase in amino acid oxidation during the exercise period. Given that endurance athletes generally consume protein at or above [36] the current recommended dietary allowance, our results suggest that the dose of protein in the present study may not be sufficient to optimize whole-body protein recovery in many athletes when accounting for exercise-induced amino acid losses; this may also be particularly relevant for athletes who train for longer than 60 min and/or perform exercise bouts with low carbohydrate availability [35], the latter of which may increase amino acid oxidation ~3-fold [21]. Thus, future investigations warrant

the examination of greater protein doses and/or more frequent feedings in order to enhance whole-body protein balance in endurance athletes under a variety of training conditions.

The duration of exercise in the present study may have also impacted net balance during recovery. From a practical standpoint, endurance athletes generally vary their training loads and/or distances, which may ultimately impact the recovery and repair of critical body proteins [35]. Based on the results from the present study, athletes who chronically engage in endurance exercise may benefit from consuming protein and carbohydrate during their training bouts in order to limit endogenous substrate loss (i.e. muscle glycogen and amino acids) and increase nutrient availability during recovery. Furthermore, a >18 g dose of protein immediately after exercise, and/or more frequent feedings throughout the day may be required to fully replace amino acid losses incurred during the exercise period.

In summary, we demonstrate that a moderate amount of protein within a mixed macronutrient beverage enhances postprandial leucine balance; however, the amount of protein consumed was not sufficient to fully replace exercise-induced oxidative leucine losses, which resulted in a negative total leucine balance during postexercise recovery. Therefore, a greater dose of protein and/or more frequent feedings may be required to counterbalance the exercise-induced oxidative losses and fully restore net leucine balance during recovery. Moreover, despite significant increases in markers of gut damage during exercise, the appearance of dietary-derived leucine was not altered during recovery in our trained men.

2.2 Figures

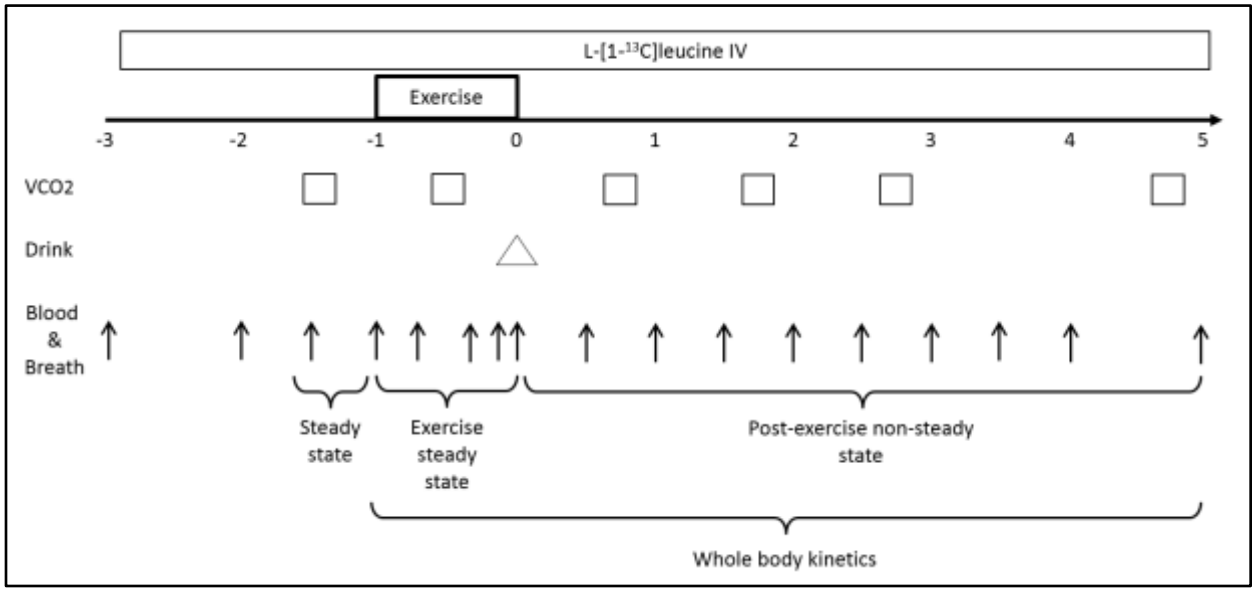

Figure 1. REST and EX trial schematic. At 0 h, participants consumed a beverage contained 20 g of intrinsically labeled egg protein and 50 g of carbohydrate at rest and after a 1 h bout of treadmill running at 60-70% VO_{2peak}. The trials differed only by the inclusion of exercise.

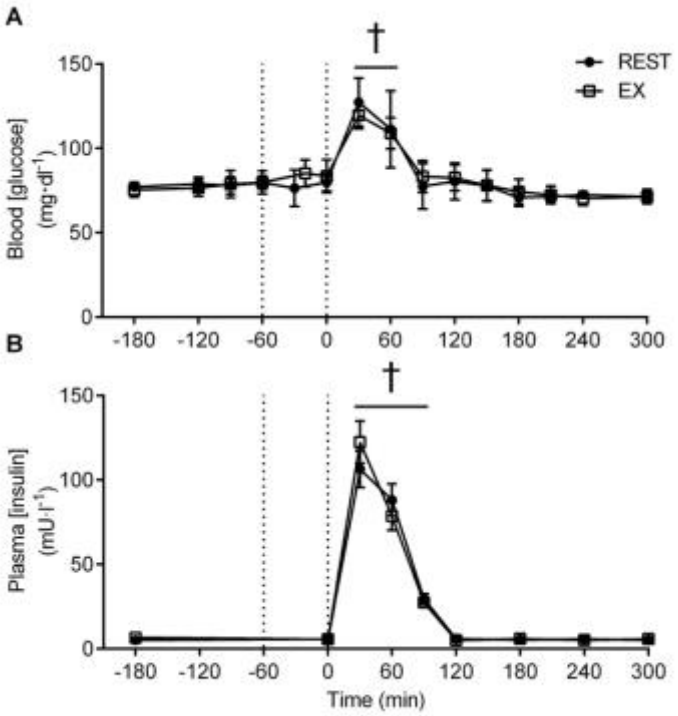

Figure 2. Blood glucose (**A**) (mg·dl^{-1}) and plasma insulin (**B**) (mU·l^{-1}) concentration. Data were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis for main effect of time. Values are mean ± SD. † denotes difference from baseline in the rest and exercise conditions ($P < 0.001$).

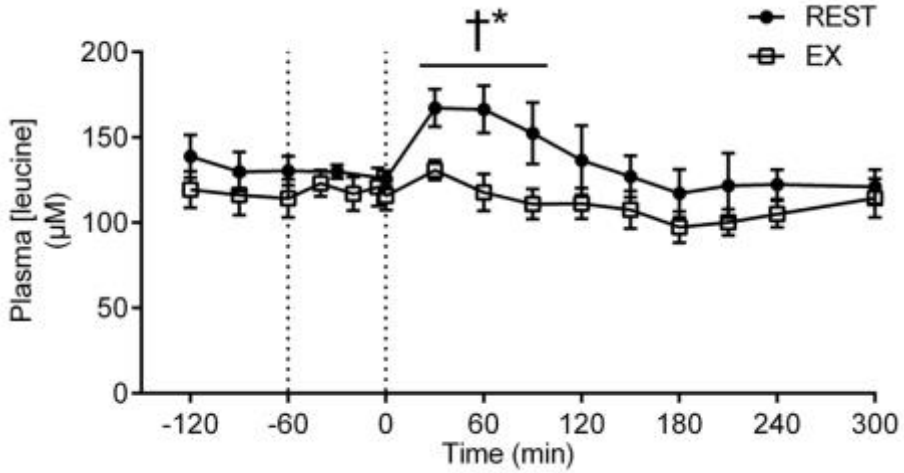

Figure 3. Plasma leucine concentration (µM). Data were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis. Values are mean ± SD. † denotes difference from baseline in the rest condition ($P < 0.001$). * denotes difference between the rest and exercise conditions ($P < 0.001$).

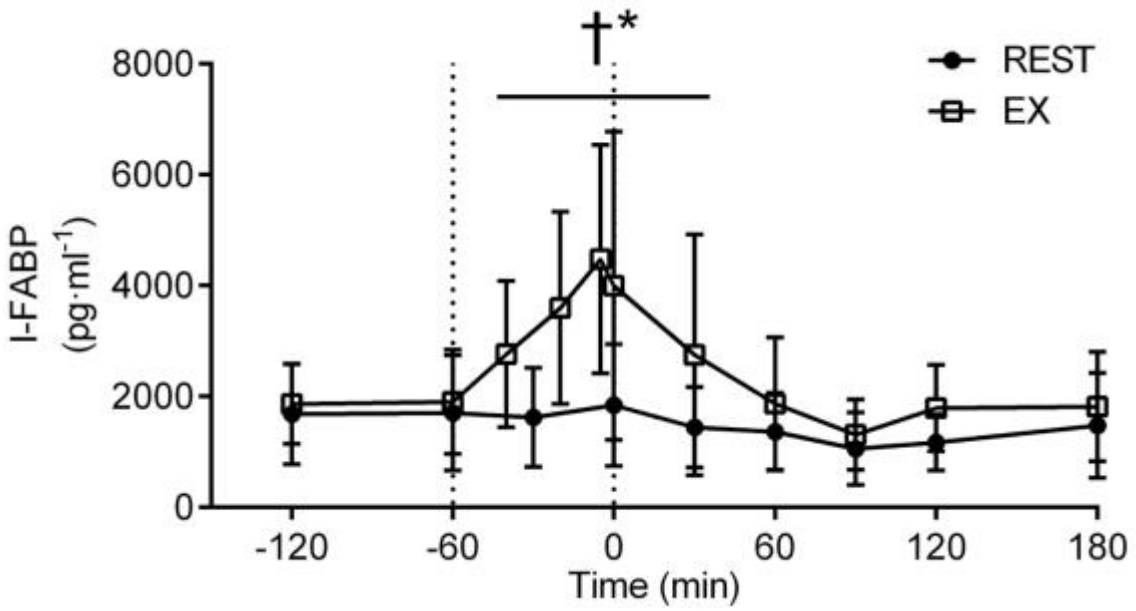

Figure 4. I-FABP concentration ($pg \cdot ml^{-1}$). Data were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis. Values are mean ± SD. † denotes difference from baseline in the exercise condition ($P < 0.001$). * denotes difference between the rest and exercise conditions ($P < 0.001$).

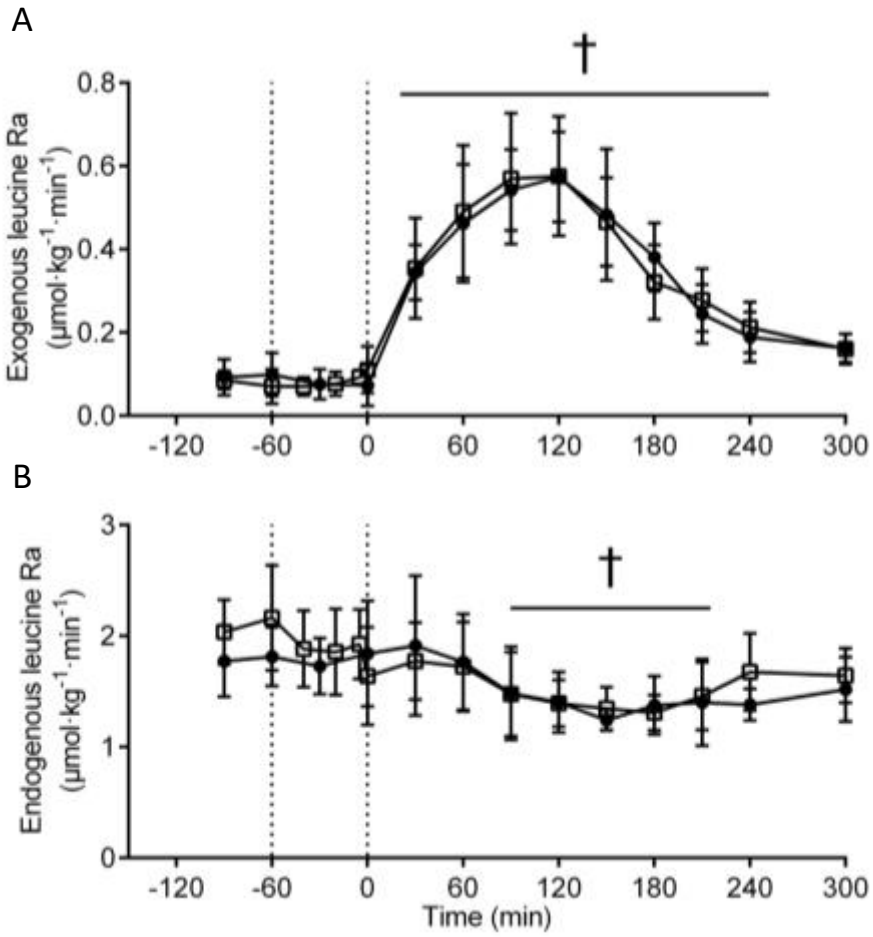

Figure 5. Exogenous (**A**) and endogenous (**B**) leucine rate of appearance (μmol·kg^{-1}·min^{-1}). Data were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis. Values are mean ± SD. † denotes difference from baseline in the rest and exercise conditions ($P < 0.001$).

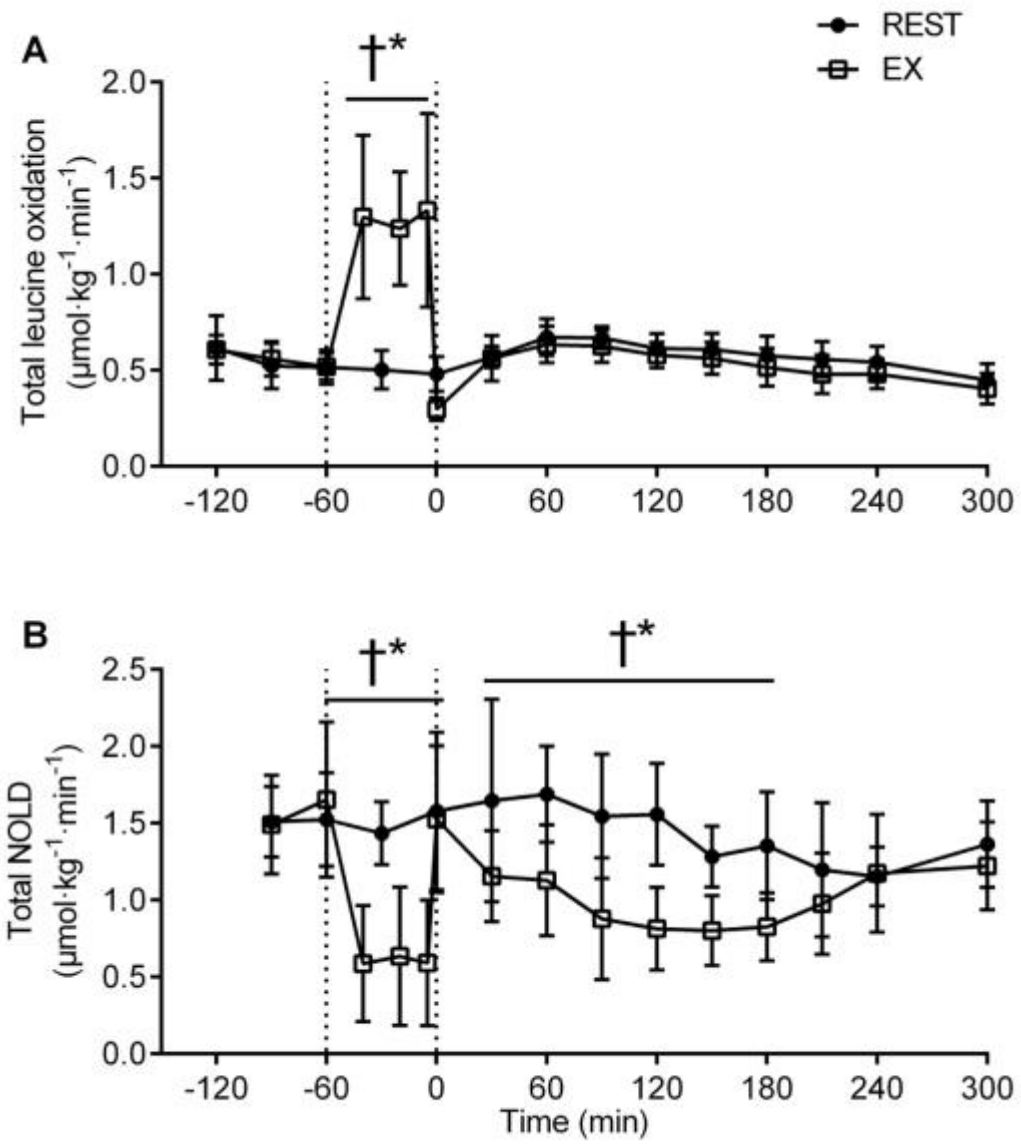

Figure 6. Rate of total leucine oxidation (**A**) and NOLD (μmol·kg^{-1}·min^{-1}) (**B**). Data were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis. Values are mean ± SD. † denotes difference from baseline in the exercise condition ($P < 0.001$). * denotes difference between the rest and exercise conditions ($P < 0.001$).

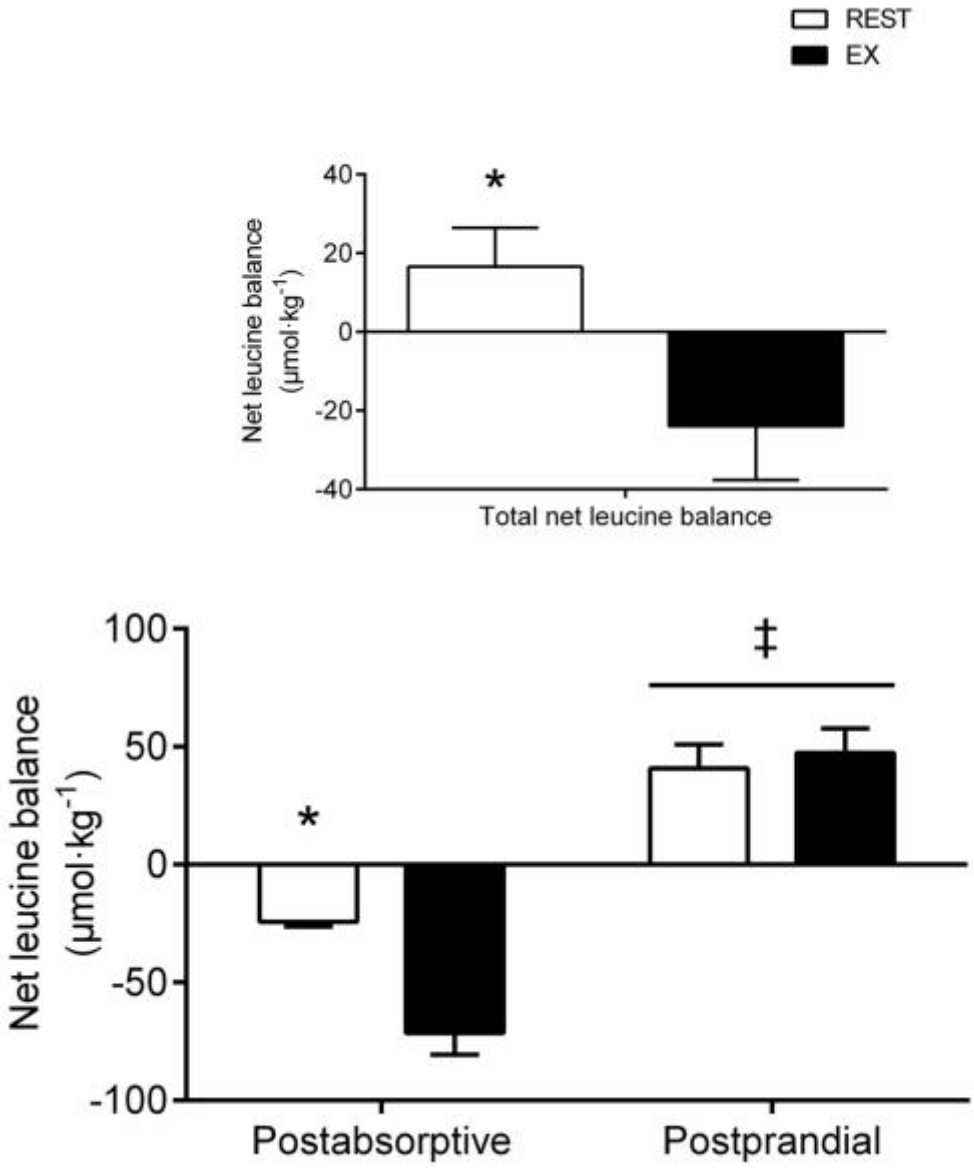

Figure 7. Net leucine balance (µmol·kg^{-1}) was calculated as the difference between leucine intake and oxidation over the postabsorptive and postprandial periods, respectively. Total net leucine balance was calculated as the sum of the postabsorptive and postprandial balances. Data for the postabsorptive and postprandial net balances were analyzed using a 2-way repeated measures ANOVA with Tukey's *post hoc* analysis. Data for total net balances were analyzed using a 2-tailed paired t-test. Values are mean ± SD. * denotes difference between rest and exercise ($P < 0.001$). ‡ denotes difference between postabsorptive and postprandial for same condition ($P < 0.001$).

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CHAPTER 3: DISCUSSION

Dietary protein and exercise are critical components for the maintenance and remodeling of skeletal muscle. Accordingly, the physiological effects of both have unique bearing on performance improvements and recovery optimization for endurance athletes. Endurance exercise has been shown to more heavily rely on amino acids as an alternative fuel source [17] than resistance exercise [18]. Dietary protein ingestion serves to offset basal and exercise-induced nitrogen losses, providing an anabolic response and improving whole-body net balance [5, 16]. The reliance on amino acids for fuel during prolonged exercise limits their availability for protein synthesis, which should be considered when determining ways to maximize recovery for endurance athletes. To date, no study has compared the effects of both rest and exercise trials on influencing dietary protein digestion and absorption kinetics, whole-body leucine kinetics, and leucine oxidation rates. Furthermore, this study is the first of its kind to utilize the novel study tool of intrinsically labeled whole eggs in this context.

The present study sought to determine the impact of 18 g of protein from whole eggs in a mixed macronutrient beverage for improving whole-body net leucine balance at rest and following an acute bout of treadmill running in trained young men. The major findings of this study demonstrate that 18 g of egg protein attenuates but does not replete oxidative leucine losses accrued during exercise. Additionally, despite significant increases in plasma I-FABP, we did not see an augmentation in dietary protein digestion and absorption kinetics. As the outcomes of this research project have been discussed at length in chapter 2, this chapter will aim to discuss noteworthy findings and practical considerations for application.

As noted in the previous chapter, our whole-body leucine kinetics data showed that feeding, but not exercise, blunted the protein breakdown response in the postprandial period. This agrees with previous findings from Knapik et al., where endurance exercise in the postabsorptive state had a negligible effect on whole-body protein breakdown [8], as well as data showing an inhibited protein breakdown response in the postprandial state [1]. Interestingly, we showed that protein synthesis was markedly more elevated in the resting condition for both the postabsorptive and postprandial states, suggesting a blunted protein synthetic response caused by exercise that was not attenuated by feeding in the postprandial period. This is further represented by a significant increase in leucine oxidation and negative net balance in the exercise condition, suggesting that the ingested protein dose was not capable of offsetting oxidative losses during exercise. It is well understood that endurance exercise causes a marked increase in the mobilization of amino acids as an alternative fuel source in order to support muscle contraction [17, 21]. However, data is limited in regard to the amount of protein that is required to offset these losses. Levenhagen et al. and Lunn et al. have both shown similar improvements to whole-body net protein balance with small amounts of ingested protein (10 g and 16 g, respectively) [11, 12]. However, in neither study was positive whole-body balance achieved. One thing to note is that this response may be intensity or duration dependent, as greater oxidative losses have been shown to occur at varying intensities and duration of exercise [3, 10, 13], and therefore should be considered when defining post-exercise recovery strategies.

Previous findings have shown that gut damage, as a result of splanchnic hypoperfusion during exercise, can augment the digestion and absorption kinetics of ingested protein [20]. We chose to measure plasma I-FABP as a marker of gut damage, and our findings are in agreement with those previously described during endurance exercise [19]. However, conversely to previous

findings, we showed there was no impairment in the appearance of dietary-derived leucine. This may be explained by the differences in ingested protein and rates of appearance into circulation. In the previous study by van Wijck et al., the rate of appearance of plasma phenylalanine peaked rapidly at 30 minutes and coincided with the return of I-FABP to normal resting values, whereas in the present study, peak rate of appearance occurred at 120 minutes, while I-FABP returned to baseline values after 60 minutes. The divergence of the two peaks may explain why we did not see an augmented response to digestion and absorption kinetics in the present study.

Our decision to choose a moderate dose of protein was twofold. Data from Moore et al. would suggest that a near-maximal rate of muscle protein synthesis can be achieved through the ingestion of 20 g of albumin protein [15], and the ingestion of a similar amount of protein has been shown to improve whole-body net balance [11]. As well, our egg beverage contained 18 g of egg protein coming from 3 whole eggs. We considered this to be representative of a normal amount of eggs that one might consume in a single sitting. The inclusion of carbohydrates to the drink makes the protein beverage more "meal-like," as protein in isolation has a faster rate of digestion and absorption than protein that is consumed with carbohydrate or fat [2, 6], and most meals are usually eaten with a mixture of the three macronutrients.

The practical applications of this data are mixed in nature. The major findings of the present study show that 18 g of egg protein is unable to offset oxidative losses of leucine and achieve a positive net balance. In the context of maximizing recovery for endurance athletes, this is important. We can see from available data that low doses of protein are likely not ideal for the individual engaging in endurance-type exercise, as the anabolic response to the ingestion of a low dose of protein does not wholly improve net balance. Yet, studies that have utilized higher doses of protein are often not consistent in terms of exercise intensity or duration. For example,

individuals from a study by Koopman and colleagues achieved a positive net balance when consuming 33 g of protein and carbohydrates in the postexercise period [9]. However, these individuals utilized a constant feeding method of protein ingestion every 30 minutes from the beginning to the end of the trial. As well, the particular aim of this study was to examine ultra-endurance athletes, which required individuals to train at a much lower intensity (45% W_{max}) for a much longer duration than what is likely representative of those performing moderate intensity endurance exercise (>60-70% VO_{2max}). And findings from Kim et al. utilized protein doses two-to-three-fold higher than what has been shown to near maximally stimulate muscle protein synthesis [7], thus potentially drowning out any subtleties in dose-response. Because the literature is mixed, it would be prudent for future research to specifically examine the dose-dependent relationship of protein ingestion and whole-body net balance in this group of individuals, or to consider frequent feeding options as a strategy for attenuating loss of leucine.

Further applications of this research might consider the training status of the individuals performing exercise when determining postexercise recovery strategies. It has been shown that endurance exercise training is capable of attenuating exercise-induced leucine oxidation [13]. Yet, conversely, the training status and dietary intake of individuals regularly performing endurance exercise show a greater requirement of dietary protein in order to offset oxidative losses [4, 14]. This may have bearing on acute postexercise recovery options when comparing competitive versus recreational athletes.

It is not yet well-known how transient gut damage during exercise may impact the digestion and absorption kinetics of dietary-derived protein in the postexercise period. Our study is the first of its kind to specifically examine this response with endurance-type exercise. One might make the argument that the divergence in peak exogenous leucine rate of appearance and peak plasma

I-FABP in our study would suggest that postexercise recovery strategies should focus on whole food protein sources, or mixed-macronutrient protein sources to achieve delayed gastric emptying. However, due to the conflicting nature of our data and that which has been previously described [20], further research may need to be performed before definitive postexercise strategies are determined for this unique situation.

In conclusion, we have demonstrated that a moderate amount of protein in a mixed macronutrient beverage is capable of improving net leucine balance, but it is not capable of offsetting oxidative leucine losses and achieving positive net balance. We have also shown that the induction of significant gut damage during exercise does not modulate digestion and absorption kinetics during recovery. It would be prudent to consider strategies such as larger protein doses or more frequent feedings to counterbalance oxidative losses and restore leucine balance during recovery from moderate intensity endurance exercise.

3.1 References

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